Sublethal effects of conventional and biorational larvicides on the physiological and biochemical status of mosquito vectors

SYNOPSIS

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Introduction

Arthropod-transmitted pathogens cause significant human mortality and morbidity throughout the world. This is especially true in developing countries where vector-borne diseases place an increasing burden on the health care system. Vector mosquitoes are capable of transmitting potential pathogens to human beings and they are responsible for several infectious diseases like malaria, filariasis, Japanese encephalitis, yellow fever, dengue and chikungunya (Nauen, 2007). They have, therefore, become a challenging problem to public health in tropical and subtropical countries. Mosquito-borne diseases are endemic over 100 countries, causing mortality of nearly two million people every year and at least one million children die of such diseases each year, leaving as many as 2,100 million people at risk around the world (Kundsen and Sloof, 1992; Weir and Stewart, 1997; Klempner *et al.,* 2007). In India, various species of *Aedes*, *Anopheles* and *Culex* mosquitoes are important insect vectors of human diseases.

Aedes aegypti, principal vector of dengue fever, is widely distributed in tropical and subtropical regions. Dengue fever incidence has increased in last years and nearly half of the world's population is now at risk (Rahuman *et al.,* 2008). According to the World Health Organization (WHO 2008 a, b), there may be over 50 million dengue cases annually and so these diseases have emerged as major international health problems. *Anopheles stephensi* is recognized as a major vector for urban malaria in India. Malaria now is responsible for the estimated more than 300 million people falling ill and there are one million deaths per year (WHO, 2007). This species prefers to breed in small man made water collections and is responsible for frequent outbreaks of malaria, particularly at construction sites in urban areas (Mittal *et al.,* 2005). *Culex quinquefasciatus* is a vector of lymphatic filariasis and it is widely distributing tropical diseases, with around 120 million people infected worldwide and 44 million people having common chronic manifestation (Bernhard *et al.,* 2003). Over 120 million people are currently infected and around 1.3 billion people in more than 80 countries are at risk of infection (WHO, 2007).

In rural and urban areas of the country vector mosquito incidence is ever increasing day by day due to the rapid urbanization and industrialization taking place in the country. This applies specially to the disposal of waste water in open sewers, stagnant and standing polluted waters which serves as an ideal breeding place for mosquito species. For this and other reasons mosquitoes present in these areas creating a vector control problem everywhere throughout the year. Attempts

to control and prevent mosquito-borne diseases since time immemorial have been met with mixed success. The most effective approach adopted for control of these mosquito-borne disease transmission either by killing or preventing mosquito bites by causing larval mortality on a large scale in breeding places.

Vector control strategies towards the end of the twentieth century emphasized the application of synthetic pesticides. As a consequence of the emergence of resistant insect populations, more number of biocontrol agents were screened for their efficacy, mammalian safety and environmental impacts. Many larvicides have been investigated as potential agents for vector mosquito control including botanical (Mulla and Su, 1999; Schmutterer, 2002; Nathan *et al.,* 2005; Lucantoni *et al.,* 2006) microbial (Goldberg and Margalit 1977; Poopathi and Tyagi, 2002; Vyas *et al.,* 2007; Mohanthy and Prakash, 2008) insect growth regulators (IGR's) (Mulla *et al.,* 1989; Ishii *et al.,* 1990; Ross *et al.,* 1994; Darriet *et al.,* 2010) and chitin synthesis inhibitors (CSI's) (Vasuki and Rajavel, 1992; Wilson and Cryan, 1997; Mulla *et al.,* 2003; Batra *et al.,* 2005).

Extensive literature survey clearly indicates that vast research work has been carried out on evaluation of various chemical and biological larvicides on different mosquito species however studies relating to their impact on biochemical profile are very scanty. Recent research has been initiated to observe larvicidal effects on some energy reserves which affect the metamorphosis of mosquito larvae. Moreover, it is evident from some papers that insecticides may pose severe effects on protein and nucleic acid which are regarded as important biomarkers of metabolic activities of cell as they play the main role in regulating the different activities of cell. Therefore, the present study is proposed to investigate a comparative picture of sublethal effects of selected conventional and biorational larvicides on physiological and biochemical status of mosquito vectors.

Review of literature

In recent years, biochemical studies of mosquito larvae have been done by several workers by using various larvicides. Massoud *et al.,* (2001) determined the biochemical changes in *Culex pipiens* treated with oil and oleo-resin extract of *Myrrh commiphora molmol* which revealed inhibitory action on the protein contents and loss of certain enzymes which effect the metabolic processes. Lerdthusnee and Chareonviriyaphap (1999) compared isoenzymes patterns of 13 fields-collected populations of *Ae. aegypti* by using starch gel electrophoresis. Three populations were collected before Bti application and ten populations were collected after Bti treatment. The results revealed that number of polymorphic loci were lowered in Bti treated populations as compared to controls.

Rivero and Ferguson (2003) determined changes in energetic (sugar, glycogen and lipids) and biochemical (protein) levels in *An. stephensi* larvae infected with malaria parasite. Rohani *et al.,* (2005) studied the protein synthesized by dengue infected *Ae. aegypti* and *Aedes albopictus*. They concluded that dengue virus was shown to induce specific protein bands in both *Ae. aegypti* and *Ae. albopictus*. Rivero *et al.,* (2006) investigated the energy budget of *Ae. aegypti* larvae infected by *Vavraia culicis*. For this purpose, they quantified the glycogen, sugars, lipids and protein reserves of infected and uninfected larvae.

Vinayagam *et al.,* (2008) studied larvicidal activity of some medicinal plant extracts against malaria vector *An. stephensi*. After the treatment with various plant extracts the larvae were taken for further evaluation of various physiological and biochemical studies like carbohydrate, DNA and RNA. Fallatah (2009) investigated histopathological and biochemical effects of myrrh, pomegranate and black seed on *Cx. quinquefasciatus* larvae and protein analysis showed changes in general protein profile of treated larvae compared to normal larvae Physiological (carbohydrate and lipid) and biochemical (Protein and Amino acid) status of *An. stephensi* larvae treated with various medicinal plants was observed by Senthilkumar *et al.,* (2009).

Begum *et al.,* (2010) evaluated the insecticidal properties of *Calotropis procera* and *Annona squamosa* ethanoloic extracts against *Musca domestica*. Bakr *et al.,* (2010 a) studied the effects of chitin synthesis inhibitors on some biological and biochemical aspects of the cotton leaf worm *Spodoptera littoralis* (Lepidoptera: Noctuidae). Results revealed significant decrease in the levels of nucleic acid and protein after 48 h of exposure. Similarly, when 6th instar larvae of *Tribolium*

castaneum were treated with higher doses of fenpropathrin, DNA and RNA contents were reduced by 20% and 21%, respectively (Shakoori *et al.*, 1996)

Several plant extracts act as insect growth inhibitors or antifeedants against a variety of insect species. Such larval intoxication and growth regulator of immature and adult mosquitoes were found correlated with some biochemical changes in the tested species particularly in a decrease or increase of the total and certain protein fraction patterns which may lead to certain functional and physiological interactions (El-Bokl, *et al.,* 1998; Mohamed and Hafez, 2000; Massoud, *et al.*, 2001 and Mohamed, *et al.,* 2003). Bakr *et al.,* (2010 b) studied the changes in protein content of *Cx. pipiens* mosquito treated with two agricultural waste extracts.

Biochemical studies on *Cx. pipipens* exposed to *Allium satvium*, *Citrus limon* and Bti was observed by Saeed *et al.,* (2010). Results revealed that the use of plant oil extracts and Bti have great effect on total protein content of treated mosquito larvae. Wisetchart *et al.*, (2011) studied the proteomics of mosquito larval gut cells treated with *Bacillus sphaericus* Binary toxin. Results revealed 25 protein spots increased and 22 spots decreased for the treated group compared with those of the control group. Preet and Sneha (2011) showed the biochemical evidence of potash alum for the control of dengue vector *Ae. aegypti*.

Keeping these facts in view, the present study was undertaken to investigate larvicidal activities of various larvicides under laboratory conditions to show their effects on biochemical and physiological status of mosquito larvae.

Objectives

- Bioefficacy of selected larvicides against freshly emerged IV instar larvae in order to ascertain sublethal concentration.
- To study the effects of sublethal exposure of selected larvicides on the growth indices and emergence inhibition.
- To evaluate the effect of sublethal exposure of selected larvicides on physiological status of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* larvae.
- To investigate the larvicidal effects on quantitative and qualitative changes in the DNA of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* larvae.
- To study qualitative and quantitative alterations in the protein of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* larvae.
- To study larvicidal effects on the proteomics of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* larvae.

Materials

Experimental animal: Freshly emerged IV instar larvae of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus*.

Mosquito species

There are over 3,000 species of mosquitoes in the world. The most common, and most dangerous, are the various species in the *Aedes, Anopheles* and *Culex* genera.

***Aedes* mosquitoes**

The mosquitoes in this genera are floodwater mosquitoes, meaning they lay their eggs on moist soil or in containers that periodically catch rainfall. They prefer to breed in tree holes, overflow ditches, and old tires. The eggs can survive drying and hatch once flooded by water. They develop in a four-stage process like other mosquitoes. As a predominantly tropical and subtropical group, *Aedes* mosquitoes tend to breed in warm weather, although some species can survive in colder environments. The adults feed day and night, and several of the species are considered particularly troublesome. Two *Aedes* mosquitoes are also carriers of dangerous disease. *Ae.*

albopictus, the Asian tiger mosquito, transmits dengue fever while *Ae. aegypti*, the yellow fever mosquito, transmits dengue, yellow fever and chikungunya.

Anopheles mosquitoes

Mosquito species in this group breed during the warmer months. Females also deposit their eggs on the surface of water in groups of 50 to 200. Over a period of about two weeks, the eggs hatch, larvae emerge, develop into pupae, and then into adult mosquitoes. However, unlike other mosquito larvae, *Anopheles* larvae do not have breathing tubes, so they must lie parallel to the surface and breath through holes in their sides called spiracles. *Anopheles* mosquitoes prefer clean water habitats in marshes, swamps, and rice fields, among others. *Anopheles* mosquitoes are the carriers of the parasite that causes malaria. More than one million deaths each year are attributed to malaria passed on by *Anopheles* mosquitoes.

Culex mosquitoes

These mosquitoes tend to hibernate over the winter and breed during the warmer months, laying rafts of eggs at night on the surface of standing water anywhere it can be found. Over a period of about two weeks, the eggs hatch, larvae emerge, develop into pupae, and then into adult mosquitoes. They normally don't travel more than a few hundred yards from where they hatched. Adults feed primarily from dusk until a few hours after dark and are considered aggressive and persistent biters, although they prefer birds to people. They can live up to a month. *Culex* mosquitoes are the carriers of the virus that can cause filariasis and Japanese encephalitis.

Larvicides selected

Selected larvicides may be grouped into two broad categories

- **Conventional larvicides**
- **Biorational larvicides**

Conventional larvicides

Conventional larvicides are human made chemicals developed and manufactured specifically for use as pesticides**.** The following conventional pesticide will be used for the study.

Permethrin

Permethrin belongs to chemical class pyrethroid. Permethrin is a broad spectrum synthetic pyrethroid insecticide, used against a variety of pests, arthropod vectors and various types of flies etc. Chemical formula of permethrin is $C_{21}H_{20}Cl_2O_3$. The International Union of Pure and Applied Chemistry (IUPAC) name for permethrin is 3-phenoxybenzyl,2-dimethyl-cyclopropanecarboxylate. Permethrin is known to act as neurotoxin.

Biorational larvicides

The term biorational is derived from two words, biological and rational. They are substances that are biologically rational or logical, that when used for specific pests have very limited or no affect on nontarget organisms. The following biorational larvicides will be used for the study. Biorational pesticides can be classified into two categories.

- **Biochemical**
- **Microbial**

Biochemical

Pyriproxyfen

Pyriproxyfen is a broad-spectrum insect growth regulator with insecticidal activity against public health insect pests. It is a WHO recommended insecticide for the control of mosquito larvae. In agriculture and horticulture, pyriproxyfen has registered uses for the control of scale, whitefly, bollworm, jassids, aphids and cutworms. Pyriproxyfen is a pyridine based pesticide and chemical formula is $C_{20}H_{19}NO_3$.

Triflumuron

Triflumuron is very widely used chitin synthesis inhibitor. It inhabits the moulting stages of larvae and prevents to develop as a proper adult. It is used to control Lepidoptera, Psyllidae, Diptera, and Coleoptera insects. Also used against larvae of flies, fleas and mosquito in public and animal health. The chemical formula is $C_{15}H_{10}C_lF_3N_2O_3$.

Neemarin

The neem tree, *Azadirachta indica*, is a native tree of India, especially in semi-arid conditions. Neemarin is a botanical larvicide derived from neem tree, where it has been used for centuries to control insects and wide range of pests. Neemarin formulation is a botanical pesticide based on neem seed kernel ingredients emulsifiable concentrate formulation. The active ingredient present in neemarin is Azadirachtin alongwith triterpenoids and limenoids. Neemarin formulation acts on the insects through multiple actions as repellent, antifeedant, insect growth regulator and oviposition deterrent.

Microbial

Bacillus thuringiensis* var.*israelensis

Bacillus thuringiensis var.*israelensis* is a bacterium which occurs naturally in soils and aquatic environments globally. In 1976, Goldberg and Margalit (1977) isolated *Bti* from *Cx. pipiens* collected in an Israeli riverbed. Since 1982, it has been used successfully worldwide as a biological pest control agent to combat mosquitoes and black flies. Bti produces toxins which are effective in killing various species of insect larvae and flies, while having almost no effect on other organisms. Indeed this is one of the major advantages of *B. thuringiensis* products in general is that they are thought to affect few non-target species.

Methodology

Study area

The study will be carried out in Agra (27°10’ N, 78°05’E) a semi arid zone of Northern India which is situated in the extreme South-West corner of Uttar Pradesh.

Larval sampling

Mosquito larvae will be collected from various localities of Agra and all instars will be maintained in the laboratory at a temperature of 25±2°C, relative humidity of 70±5% and photoperiod of 14:10 (light:dark). Larvae will be reared in dechlorinated water and fed upon 5% glucose.

Larvicidal bioassay

Larvicidal bioassays will be carried out according to the guidelines given by World Health Organization (WHO 2005). The larvicidal tests will be conducted in 250 ml of deionized water. For each test concentration 20 fourth instar larvae will be used. For experimental treatment the stock solutions of various larvicides will be prepared. To find out the effects of various larvicides on mosquito larvae at first we set up wide range of test concentrations and LC_{50} will be calculated. From these results we will determine the sublethal concentrations for experimental set up. Each experiment will be conducted in five replicates and for each experiment control will be run as negative test concentration. After giving the 24 hours of sublethal exposure fourth instar larvae will be collected from experimental and negative test concentrations to determine the content of nutrient reserves (glycogen, sugar and lipid) and biochemical (protein and DNA) to determine the possible mode of action leading to the larval mortality. To carry out the biochemical studies five mosquito larvae will be taken for each assessment.

Percent pupation

Percentage cumulative pupation and the mean larval period will be calculated by using following formula

$$(A*1) + (B*1) +(C*1) + (D*1) + (E*1) +\ldots\ldots\ldots\ldots..+ (H*1)$$

Where A, B, C, D……..H are the number of pupae will be collected on the 1,2,3,4 …. On the respective days.

Emergence inhibition

Percentage emergence inhibition will be calculated as **100-A**. where A will be the % successful emergence.

Microseparation and analysis of nutrient levels

Micro separation of glycogen, sugar and lipid from the same mosquito larvae will follow as described by Van Handel (1965). Five mosquito larvae from each treatment allocated to the quantification of glycogen, sugar and lipid will be transferred to eppendorf with 200µl sodium sulphate solution and crushed with plastic pestle. To this homogenate 1.6 ml of chloroform-methanol (1:1) solution will be added and centrifuged for 2 min at 3,000 rpm. Supernatant will be

taken out into fresh microcentrifuge tube and pellet will be retain for glycogen analysis. Now, water will be added to supernatant and after brief centrifugation solution will separated into two fractions pellet portion for lipid analysis and aqueous layer for sugar analysis.

Glycogen analysis

For glycogen analysis anthrone reagent (5 ml) will be added to the pellet, and heated for 20 minutes at 90°C, and then samples will be cooled to room temperature. The absorbance will be read at OD_{625} nm (Van Handel 1985a). Glycogen concentrations will be obtained from the standard graph made from the glucose.

Sugar analysis

For sugar analysis the solvent will be evaporated in a heating block and the residue will reheated with 5 ml anthrone reagent (Van Handel 1985a). Then samples will be cooled to room temperature, and read at OD_{625} against blank. Sugar concentrations will be obtained from the standard graph made from the glucose.

Lipid analysis

For lipid analysis the solvent will be evaporated completely in a heating block and sulphuric acid (200 µl) will be added to the tubes and get reheated for 10 minutes at 90°C. Then vanillin-phosphoric acid reagent (5 ml) will be added (Van Handel 1985b).Samples will be cooled to room temperature to develop pink color, and read at OD_{525}. Lipid concentrations will be obtained from the standard graph made from the vegetable oil.

Microseparation and analysis of biochemical levels

DNA extraction

DNA from mosquito larvae will be extracted by the method of Ballinger- Crabtree *et al.*, (1992) with slight modification. Mosquito larvae from each treatment will be ground in 200 µl lysis buffer (100 mM Tris –HCl, pH 8.0, 1% Sodium dodecyl sulphate, 50 mM NaCl, 50 mM EDTA) and the mixture will be treated with 5 µl of proteinase K (20 mg/ml) for 4 hours at 56°C. The suspension will be extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and twice with chloroform-isoamyl alcohol (24:1) and DNA was precipitated by the addition of 0.2 volumes of 5M NaCl and 2.0 volumes of absolute ethanol. The mixture was incubated overnight at

-20°C and spun at 12,000 rpm for 20 min to get pellet which was resuspended in 100 µl of TE buffer and stored at 4°C.

Quantitative and Qualitative analysis of DNA

The extracted genomic DNA would be subjected to quantification using UV- visible spectrophotometer. Absorbance will be measured at OD_{260} and OD_{280} and the ratio will be calculated. DNA samples will be checked for intact DNA on 1% agarose gel electrophoresis.

Protein extraction

Total protein will be extracted from treated and untreated larvae following homogenization of the sample using a pestle grinder according to the T-PER extraction kit (Pierce, Tissue protein extraction reagent).

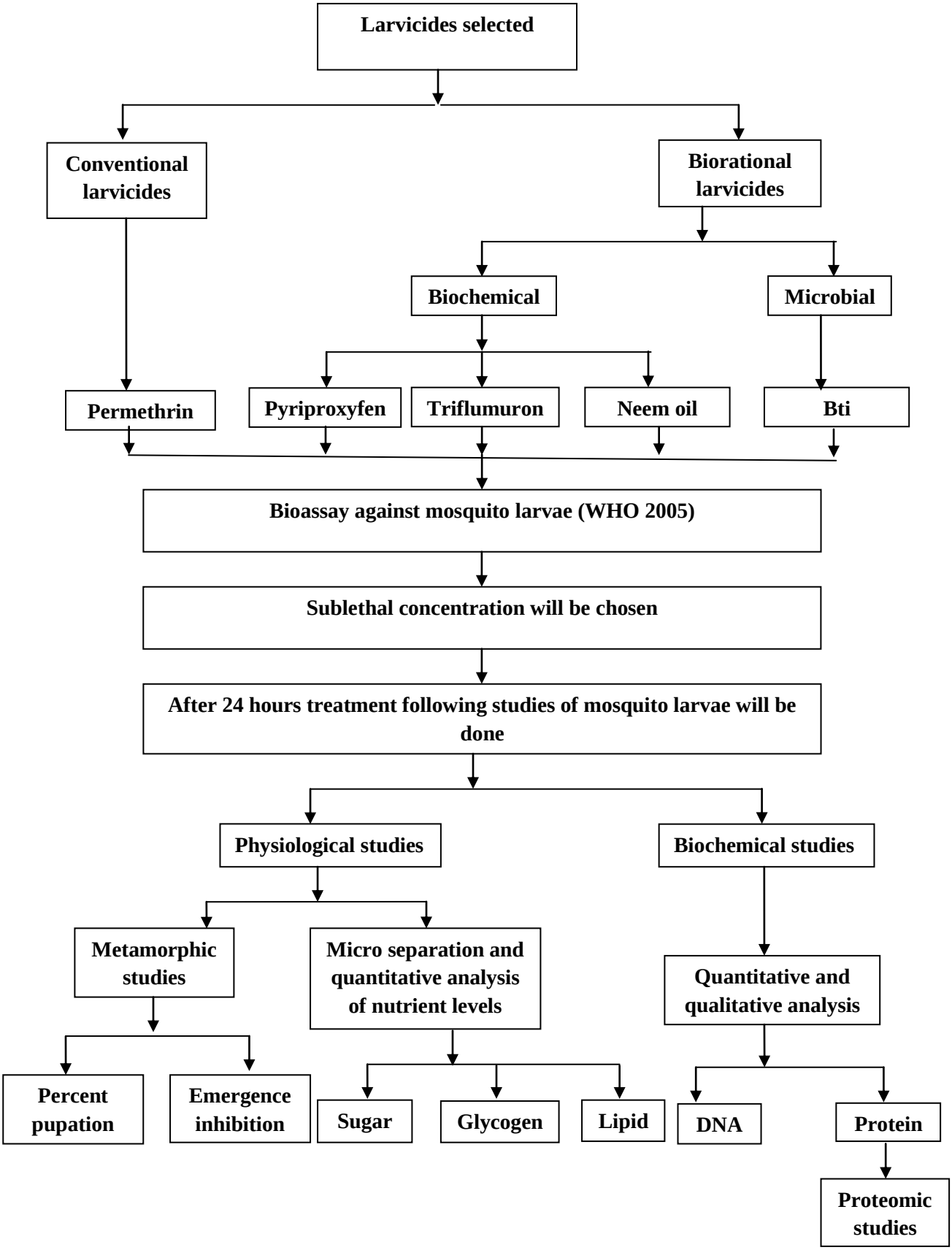
Quantitative and Qualitative analysis of Protein

The extracted protein sample would be subjected to quantification according to Pierce BCA protein assay kit. Quantitative alterations in protein samples would be done by SDS-PAGE. Some selected samples from treated lot will be subjected to further proteomic analysis.

Statistical analysis

Results of biochemical studies will be analysed statistically and quantitative estimation of sugars, glycogen, lipids, proteins and DNA content will be done with the help of regression equation. The data will be presented as mean ± SD value. Annova will be used to analyse mean difference between control and treated groups.

Experimental Design



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